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In Situ Biodegradation of Nitroaromatic Compounds in Soil

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In Situ Biodegradation of Nitroaromatic Compounds in Soil

Introduction

Nitroaromatic compounds, particularly nitrotoluenes used as explosives and nitroaromatic herbicides such as dinoseb, are serious environmental contaminants at industrial locations nationwide. Research performed during the 1970s (15, 18) generally indicated that complete biomineralization of 2,4,6-trinitrotoluene (TNT) and similar highly nitrated compounds did not occur. Biological reductions ($R\text{-}NO_2 \rightarrow R\text{-}NO \rightarrow R\text{-}NHOH \rightarrow R\text{-}NH_2$) and polymerization reactions appeared to occur, but actual degradation of aromatic nuclei was not observed. However, this work involved studies of aerobic systems such as activated sludge and thermophilic composts, and pure culture studies of aerobic fungi and bacteria such as pseudomonads. Pure cultures of some anaerobic bacteria such as *Veillonella alcalescens* (35) were examined, with similar results. Boopathy and Kulpa (2) recently isolated a *Desulfovibrio* that used TNT as a sole source of nitrogen, producing toluene as an end product. A *Pseudomonas* that produced dinitrotoluene, mononitrotoluene, and toluene from TNT, perhaps by hydride additions, was isolated by Duque et al. (10). These are still incomplete degradations of the parent molecule. Since the *Desulfovibrio* strain required obligately anaerobic conditions to produce toluene from TNT, and the *Pseudomonas* did so aerobically, the TNT transformation process may be mechanistically different in the two microorganisms. This should be a fruitful area for future research.

It has recently been found that obligately anaerobic microbial consortia can mineralize many recalcitrant chemicals (toluene, chloroform, benzene, chlorophenols, etc.) that had been considered essentially nonbiodegradable in the absence of oxygen (19, 20, 21). Extensive work at the University of Idaho with obligately anaerobic microbial consortia indicates that these systems are capable of complete biodegradation of nitroaromatic pollutants. Work with 2-*sec*-butyl-4,6-dinitrophenol (dinoseb) has shown complete fermentation of this nitroaromatic pollutant in soils by anaerobic consortia without buildup of aromatic biotransformation products (16, 17). Similar results were observed with a variety of nitrotoluenes and munitions residues, including TNT and trimethylnitramine (RDX) under appropriately controlled conditions (12, 13). In the work with TNT and RDX, hydroxyaromatics were observed as metabolites of TNT. Their presence indicates a complex series of reductive and hydrolytic reactions that led to the conversion of TNT to products that can be fermented by members of the consortia. Additional work with radiolabeled TNT is required to clarify these pathways and provide overall mass balances for the various TNT biotransformation products

Degradation of Nitroaromatic Compounds by an Anaerobic Consortium

Although several anaerobic microbial systems have been described for degrading aromatic chemicals, little practical information has been available on using such cultures to bioremediate nitroaromatic-contaminated soils. The method of Funk et al. (12, 13), developed, patented, and licensed at the University of Idaho, appears to be useful for biologically remediating nitroaromatic herbicide-contaminated soils and explosives-contaminated soils, such as those from agricultural

chemical spill sites and military munitions depots (32, 33). For example, 12,000 mg/kg of TNT, 3,000 mg/kg of RDX, and 300 mg/kg of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) were found as contaminants in a soil used as a model system to develop the technology. A consortium of anaerobic bacteria transformed these contaminants to nontoxic, nonaromatic, and aerobically mineralizable end-products, including various organic acids. We have now begun the process of characterizing the microbiological components of this bioremediation system.

When a soil inoculum from this consortium was added to a buffer solution at near neutral pH containing a soil from a U.S. Army munitions site near Umatilla, Oregon, TNT was completely removed within 5 days, and RDX within 24 days (13). We tracked the fate of the TNT molecule to reduced aminonitro compounds, then to *para*-cresol and other yet to be identified intermediates. Nonaromatic volatile organic acids, such as acetate, were observed as ultimate products of anaerobic TNT degradation.

To investigate the pathway for the strictly anaerobic microbial degradation of TNT, an anaerobic bench-top reactor, initially established with a sewage sludge inoculum in 1989, was supplemented periodically with a mixture of TNT and other munitions compounds as a sole carbon source. The feed contained mostly TNT, but also significant amounts of RDX and HMX, and trace amounts of many other munitions-derived compounds. These compounds were supplied in a defined medium containing essential inorganic nutrients. As the culture, a consortium of anaerobes, adapted over time to utilize the TNT, the reactor went through sequential stages of adaptation, characterized by the accumulation of specific TNT metabolites identified by HPLC, GC/MS, and ^{14}C-radiolabeled tracer studies (Funk et al., 1993a).

The first metabolites to accumulate in culture supernatants were the reduced intermediates 4-amino-2,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene. A second stage of adaptation led to the disappearance of these intermediates and the accumulation of 2,4,6-trihydroxytoluene. In a third stage of adaptation, 2,4,6-trihydroxytoluene gave way to *p*-cresol (4-methylphenol). After three years of selective pressure for growth on munitions compounds, the reactor accumulated acetate in the supernatant and the headspace as the first identifiable nonaromatic TNT degradation product. This adapted culture was used to establish optimal conditions to maximize TNT degradation rates. When fed the munitions mixture (final TNT concentration, 125 mg/liter), the reactor accumulated detectable amounts of *p*-cresol in 14 days. This bench-top reactor has proven useful in verifying the pathway of anaerobic TNT degradation to mineralizable products. The probable pathway is presented in Figure 1. Since this pathway is based only on results of characterizations of TNT metabolites, it must be considered as tentative. It remains to be determined which metabolites represent primary pathway intermediates, and whether multiple pathways are occurring simultaneously. Future studies with pure cultures isolated from the consortium will help answer these questions.

We also examined the culture parameters that affect the anaerobic bioremediation of soils contaminated with TNT, RDX, and HMX, in efforts to optimize the physical and chemical conditions promoting their degradation. We flooded the soils with 50 mM phosphate buffer, added starch as a supplemental carbon substrate, and incubated the soils under static conditions. Aerobic heterotrophs, naturally present in the soil or added as an inoculum, quickly removed the

oxygen from the static cultures, creating anaerobic conditions. As observed previously, TNT was removed from the soil cultures by the strictly anaerobic microflora within 4 days. The reduced intermediates formed from TNT and RDX were removed from the cultures within 24 days. Optimal conditions were pH, 6.5 to 7.0; temperature, 25 to 35°C, nitrogen source, 25 mM; and redox potential, -100 to -300 mV. Degradation rates were improved when 25 mM ammonium chloride was added to cultures buffered with 50 mM potassium phosphate, but 50 mM ammonium phosphate buffer completely inhibited TNT reduction. When soils were incubated under aerobic conditions or under anaerobic conditions at alkaline pH, the TNT biodegradation intermediates polymerized. Polymerization was not observed at neutral to slightly acidic pH under anaerobic conditions. Polymerized TNT compounds are insoluble in the aqueous phase, so it is crucial to determine whether true degradation of TNT is taking place, or if only simple products of polymerization, which are still toxic or mutagenic, are being formed. The completion of the first stage of remediation of munitions-contaminated soils resulted in aqueous supernatants that contained no munitions residues or amino-aromatic compounds (13).

Our early work with dinoseb, a nitrophenolic herbicide commonly found as a soil contaminant, showed that under microaerophilic conditions, it is transformed to persistent multimeric forms that remain toxic, while under well-aerated conditions, no degradation occurs (29). However, in studies pre-dating our munitions work, we enriched an anaerobic consortium that fermented dinoseb and other nitroaromatic compounds under methanogenic conditions (16, 17). These initial observations ultimately led to our treatment of soils containing complex mixtures of TNT, dinitrotoluenes, mononitrotoluenes, nitrobenzoates, and related compounds (33), which showed that all contaminants could be removed to below detection limits of gas chromatography/mass-spectrometry. Even though biological treatment of several of these compounds in well-aerated cultures has been described, many of them are subject to polymerization reactions under microaerophilic conditions, which are almost certain to occur in soil treatment systems that are not maintained absolutely anoxic (18).

We have determined (17) that the optimal *in vitro* conditions for dinoseb biodegradation differ slightly from those for TNT: carbon source (carbohydrates, particularly starch), 5 g/liter; nitrogen source (ammonium chloride), 4 g/liter; temperature, 25°C (range 10-40°C); pH, 7.0-7.5; and redox potential, <-200 mV (range -200 to -500 mv). Data collected during a demonstration project in which 40 tons of dinoseb-contaminated soil underwent bioremediation indicated that the dinoseb level was reduced to below the analytical detection limit (0.5 mg/kg) in less than 25 days (32). Summary data were reported in a U.S. Environmental Protection Agency Superfund Innovative Technology Evaluation bulletin (32).

Isolation of Pure Cultures That Degrade TNT

Pure cultures of obligate anaerobes that degrade TNT have now been isolated by three researchers in our group. A gram-positive, rod-shaped bacterium capable of growth in nutrient media containing 30 ppm TNT and 50 ppm RDX (27) was shown to degrade TNT and RDX sequentially in a manner similar to the one previously described for our consortia. TNT was removed first, followed by RDX. An API AN-IDENT culture identification test indicated

Clostridium bifermentans as the closest match, an identification supported by urease and motility tests and an rRNA gene sequence. Other *Clostridium* spp. are known to be able to reduce nitroaromatic compounds (25, 26), but do not catabolize TNT beyond simple reduction pathways. The TNT and RDX pathways employed by *Clostridium bifermentans* have yet to be elucidated.

Biodegradation of TNT by a strictly anaerobic bacterial co-culture was originally observed in our lab in 1993. The co-culture was isolated from our three-year-old anaerobic bioreactor that had been fed only munitions compounds, by streaking samples from the bioreactor onto agar plates containing a mineral salts medium plus 100 ppm TNT. Preliminary evidence suggested that the co-culture degrades TNT fermentatively, producing volatile organic acids, and that it probably metabolized TNT efficiently only when a co-substrate (e.g., glucose or yeast extract) was provided (unpublished data). Apparently the agar in the original isolation medium provided contaminating nutrients sufficient for formation of colonies whose growth was primarily at the expense of TNT.

The co-culture consisted of two gram-positive, strictly anaerobic rods with heat-resistant endospores, apparently a *Clostridium* species and another similar coccoid bacterium. The culture grew on plates as a mixture of white and clear colonies, but both colony types contained co-cultures of the two cell types, as shown by scanning electron micrographs (SEM) of a white colony, and transmission electron micrographs (TEM) of a clear colony. As described below, co-cultures obtained from stocks derived from both the white and clear colony types behaved similarly in TNT degradation experiments (28). Very recently, working with the white co-culture, we have successfully separated the two cell types from one another.

To examine TNT degradation by the co-cultures, we used standard techniques employing anaerobic serum bottles (28). For the degradation experiments, a vitamin-supplemented minimal medium (11) was used, with or without additional starch (2% wt/vol). Slurried TNT-contaminated soil from the Umatilla, Oregon, site was used in place of pure TNT. Initial TNT concentrations were determined after dissolution of TNT from the soil matrix. Residual TNT and the appearance and disappearance of TNT degradation intermediates were monitored by HPLC. The co-cultures degraded TNT to nonaromatic products within 24 hours, in a process stimulated by starch. Co-cultures from both white and clear colonies were separately examined for TNT degradation in the presence and absence of starch. Use of killed controls allowed us to examine whether TNT biodegradation in the Umatilla soil could be mediated by enrichment of the natural microflora, or whether inoculation with the TNT-degrading clostridial culture was required for TNT degradation.

The results of this experiment are summarized in Figure 2. The autoclaved, uninoculated soil (sample A) and the formaldehyde-killed uninoculated soils (sample B) showed no TNT degradation, nor did the nonsterile Umatilla soil, even upon addition of starch as a cosubstrate (sample D). Thus, this soil did not contain an anaerobic microflora that could be readily enriched to degrade the TNT, a conclusion we have reported in the past (13). Previous bioremediations of Umatilla soil have always involved inoculation of the soil with a competent microflora from another soil. When nonsterile soil was inoculated with either the white or clear clostridial co-cultures, we observed TNT degradation, which was generally more rapid in the presence of added starch. In recent experiments we have found that a 10% clostridial inoculum with a 3% (w/v)

addition of starch resulted in complete removal of 100 ppm TNT from the soil in 24 hours at room temperature. Transient intermediates detected as a result of TNT degradation include 4-amino-2,6-dinitrotoluene (4A-2,6-DNT) and 2,4-diamino-6-nitrotoluene (2,4-DA-6-NT). These intermediates accumulated during the early phases of TNT degradation, but were then removed. Thus, both of the clostridial co-cultures were capable of bioremediating Umatilla soil containing 100 ppm of TNT. Higher concentrations have not yet been examined. However, soils from Weldon Spring, Missouri, containing 2000 or more milligrams TNT per kilogram were treated successfully in field trials employing anaerobic consortia (33). Since the solubility of TNT in aqueous solution is about 100 mg/liter, it must be solubilized as it is degraded. Thus, very high concentrations of TNT (above solubility) apparently do not present a problem in terms of toxicity to the clostridia in our system.

We have also examined the bioconversion of tetramethlyenetetranitramine (HMX) and trimethylenetrinitronitramine (RDX), which are aliphatic compounds common in explosives. After demonstrating a sequential bioconversion of, first, TNT, and then RDX by an anaerobic consortium (13), we investigated this sequential process further (27), beginning with the isolation from our mixed munitions-degrading consortium (12, 13) of an obligately anaerobic bacterial isolate capable of removing RDX from its environment. Of several dissimilar isolates, the one chosen for further study was KMR-1, a strain of *Clostridium bifermentans* that degraded the most RDX in 72 hours. Electron micrographs showed it to be a pure culture. The organism, a motile, urease-negative, gram-positive, anaerobic bacillus, is apparently similar in general properties to strains previously isolated by our group. The growth of strain KMR-1 was monitored by OD at 600 nm in the presence of explosives and different reducing conditions. In nonreduced media containing no explosives, strain KMR-1 grew rapidly and maintained a very high OD, while on media containing explosives, it grew much more slowly, regardless of the reductant. In all cases there was a significant lag time in strain KMR-1 grown in media containing explosives. Data on the biotransformation of explosives by strain KMR-1 during growth in explosive-containing media under different reducing conditions are shown in Figures 3-5.

In all three media, the transformation of TNT occurred prior to the transformation of RDX. Cell numbers did not increase until TNT concentrations approached their minimum. Once the TNT concentrations dropped, an increase in cell density occurred, with concomitant co-metabolism of RDX. As the concentration of RDX reached its minimum, the cell density in the culture reached a maximum. In abiotic controls, concentrations of RDX did not decrease in any of the reducing treatments, but concentrations of TNT decreased in all treatments. A trend was seen in the amount of abiotically transformed TNT and the theoretical E_h of the media. The more reduced the medium, the greater the amount of abiotic reduction of TNT. In all treatments the amount of biotically transformed explosive was greater than the amount of abiotically transformed explosive.

Funk et al. (13) reported that the biotransformation of TNT occurred over an experimental time frame of 4 days, and RDX over 24 days. This long period required for the observed biotic transformation may be due to the limited amount of growth substrate supplied to the consortium in these experiments. The comparatively short time required for biotic transformation of TNT and RDX in the pure culture, 4 hours and 23 hours respectively, may be due to the rich substrate supplied to strain KMR-1. In both these groups of experiments (13, 27), the biotransformation of

TNT took place before that of RDX, separated by a short lag time of approximately 2 hours. This time lag may represent the time required for the bacteria to develop the cellular machinery to biotransform the RDX. Thus, our accumulated evidence indicates that the organisms responsible for the biotransformation of both RDX and TNT in our enrichments probably are *Clostridium bifermentans* and closely related strains. The bioconversion of RDX and TNT occurs under anaerobic conditions both in the consortium and in pure culture without the need of an added reductant, and TNT is degraded before RDX. The presence of a readily metabolizable carbon source accelerated both biotransformations. *Clostridium bifermentans* was readily and reproducibly isolated from all our enrichments, which contain very high numbers of clostridial endospores (9, 27).

Physiology of Anaerobic TNT Biodegradation

As discussed above, an anaerobic TNT-degrading mixed culture was established by combining 1 liter of an anaerobic, dinoseb-degrading microbial consortium (29) with fresh anaerobic sewage sludge. Neither the sludge nor the enrichment could transform TNT beyond reducing the 4- and 2-nitro groups to amino groups. The consortium was maintained by periodic feeding with a munitions feed solution that contained TNT, RDX, and HMX as the major contaminants (13), and allowed to acclimate to the munitions compounds for 13 months. Intermediates other than the amino-substituted toluenes accumulated sequentially, e.g., methyl phloroglucinol and *p*-cresol (12). Fresh anaerobic sludge was added to the culture twice during the first year of acclimation to restore a lost methanogenic population. The consortium appeared to stabilize after the first year. Removal of the amino-substituted intermediates to below detectable limits usually occurred within 18 to 28 days of feeding. After two years, the consortium was modified by adding a portion to fresh sewage sludge and fresh medium (11). At the same time, fresh medium was added to the original consortium. Both consortia continued to produce methane from the munitions feed. The subenrichment that had received sewage sludge was used for the isolation of culture LJP-1.

Through electron microscope examinations, LJP-1 originally appeared to be a co-culture of two heat-resistant bacteria, a rod and a coccus. It was purified to a single strain, the rod-shaped bacterium, by heat treatment at 82°C for 20 min. The bacterium was a gram-positive (negative in older cultures), non-motile, strictly anaerobic rod with heat-stable spores, identified as a *Clostridium* strain (8).

Experiments were performed to determine whether LJP-1 could grow in a defined medium containing TNT as a sole carbon and nitrogen source. A control without TNT showed no increase in cell numbers after 24 h, while the culture with 50 ppm TNT showed an increase from 10^3 to 10^5 cells/ml 24 h after inoculation, when the cells appeared largely as spores. The aqueous phases of the cultures were assayed by HPLC (20). LJP-1 produced and then removed several major TNT degradation intermediates, including 4-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, and 2,4,6-trihydroxytoluene, within 24 h after inoculation. No nitroaromatic intermediates were seen after 24 h. The predominant organic acids present after 24 h were isovaleric, heptanoic, isocaproic, valeric, butyric, isobutyric, and formic acids. ^{14}C from ^{14}C-TNT was found in these fatty acids (unpublished data).

This was our first observation of a defined, obligately anaerobic culture that could degrade TNT provided as a source of carbon and nitrogen. The culture converted TNT to a mixture of volatile organic acids and a small amount of carbon dioxide via a fermentative pathway apparently differing from that of most *Clostridium* species by producing at least five different organic acids from the seven-carbon TNT molecule.

Transformation and Degradation of Munitions Compounds by Strict Anaerobes: Overall Perspective

If we examine all of the available information concerning transformation and degradation of nitroaromatic compounds by strict anaerobes, some emerging patterns can be seen. Work by Boopathy and Kulpa (2), Boopathy et al. (4-7), Preuss and Rieger (this volume), and Preuss et al. (25) shows that sulfate-reducing bacteria such as *Desulfovibrio* strains can use 2,4,6-trinitrotoluene as an electron acceptor and/or nitrogen source while using compounds like lactate or pyruvate as carbon sources. TNT is reduced by these strains sequentially through monoamino- and diamino-nitrotoluenes to triaminotoluene (TAT), which may accumulate or be reductively deaminated to toluene. Methanogenic bacteria also reduce nitrophenols (14) and TNT (4). Our work indicates that an alternative route for TNT degradation involves the ubiquitous reductions to monoamino-, diamino-, and probably triaminotoluene, but subsequent reactions involve hydrolytic displacements of the amino groups to form polyphenols. Polyphenols are then reductively dehydroxylated to yield simpler phenols like *p*-cresol. These alternative routes appear prominent among *Clostridium* strains isolated by enrichments on munitions compounds. These patterns are summarized in Figure 6.

There is a great deal of work still required to firmly establish the centrality among anaerobes of the pathways in Figure 6. Very few enzymes have been examined, though there have been some exciting initial studies of TNT and DANT reductases in some systems (Preuss and Rieger, this volume). The complexities of electron transport to TNT and various other intermediary substrates need attention. Many intermediates still must be identified; for example, those between triaminotoluene and trihydroxytoluene in the clostridial systems, and between triaminotoluene and toluene in the *Desulfovibrio* strains. A possible mechanism for the non-enzymic conversion of TAT to trihydroxytoluene is shown in Figure 7. We have mentioned the likelihod of this conversion in a previous publication (13). In theory, all these reactions would be reversible, but the equilibirium would be expected to favor almost complete conversion to the deaminated products. Thus, such a process may explain how TAT is metabolized further under anaerobic conditions thorugh the involvement of some non-enzymatic, but physiologically productive, reactions.

Mass balances of conversion of TNT to final products are still largely missing from the literature. How our *Clostridium* strains ferment compounds like *p*-cresol to mixtures of volatile organic acids is not known. Fortunately, with the variety of pure cultures now available, progress on these questions should be rapid. Techniques of modern enzymology (e.g., capillary electrophoresis to identify TNT-induced proteins) and molecular biology (e.g., transposon mutagenesis to produce blocked pathway mutants) should be particularly productive. Finally, we should not be surprised to encounter additional variations on TNT catabolism not suggested by work to date.

Other important munitions contaminants receiving study are trimethylenetrinitronitramine (RDX) and tetramethylenetetranitramine (HMX). Strict anaerobes appear to degrade these compounds efficiently (18, 22, 34). RDX appears to be reduced sequentially to its nitroso derivatives, and formaldehyde and methanol seem to be biodegradation products (22). The basis for the diauxic process by which both anaerobic consortia (13) and pure *Clostridium* strains (27) degrade TNT from munitions mixtures prior to RDX needs investigation. Are different reductive enzyme systems used for TNT and RDX, and are they induced sequentially? Do similar enzymes reduce both, but preferentially reduce the substrates and their partial reduction products according to their redox potentials? For a pathway summary of RDX and HMX degradation, refer to the reviews of Kaplan (1992) and Walker and Kaplan (1992) discussed above. As with TNT, pure cultures of strict anaerobes that degrade RDX are available, so progress on understanding the details of these processes should also be rapid.

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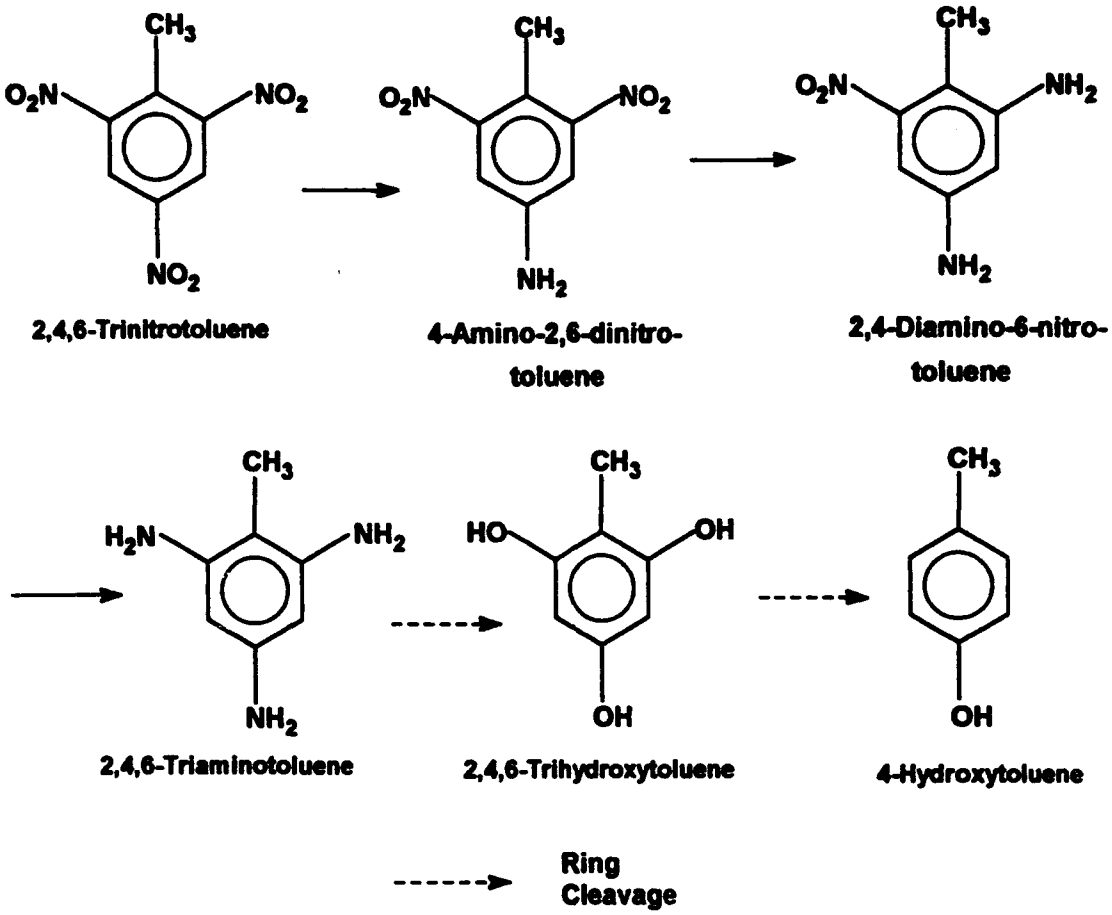

Figure 1. Proposed pathway for the anaerobic biodegradation of TNT. Solid lines, confirmed sequence of reactions; dashed lines, other, as-yet-unidentified intermediates between compounds (12).

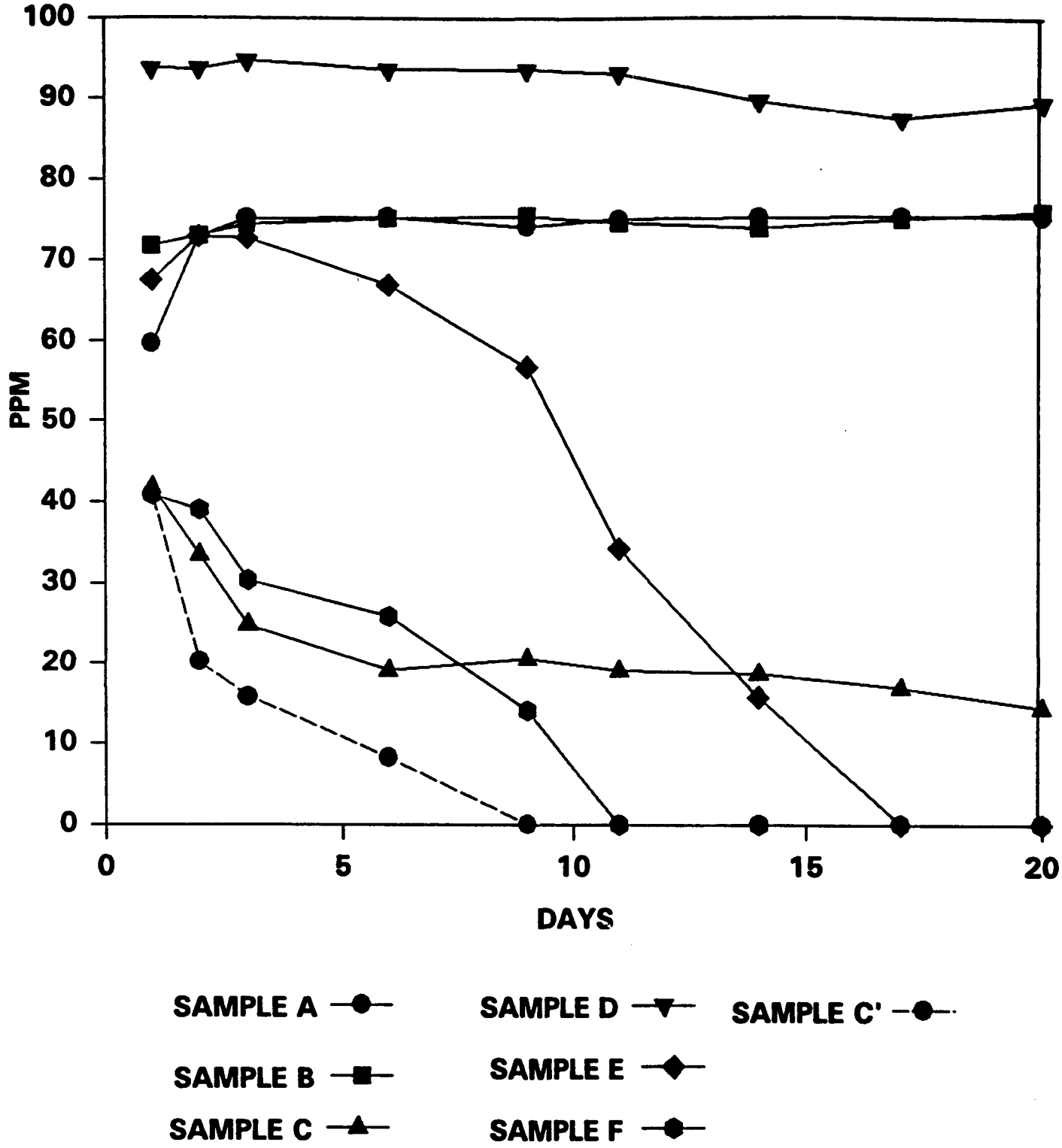

Figure 2. TNT degradation in soil by co-cultures. A (control), no inoculum, sterile (autoclaved) soil, 3% starch added. B (control), 10% inoculum of white colonies killed by 10% formaldehyde, sterile soil, 3% starch added. C, 10% inoculum of white colonies, sterile soil. D (control), no inoculum, non-sterile soil, 3% starch added. E, 10% inoculum of clear colonies, sterile soil. F, 10% inoculum of clear colonies, sterile soil, 3% starch added. C' (dashed line), 10% inoculum of white colonies, sterile soil, 3% starch added.

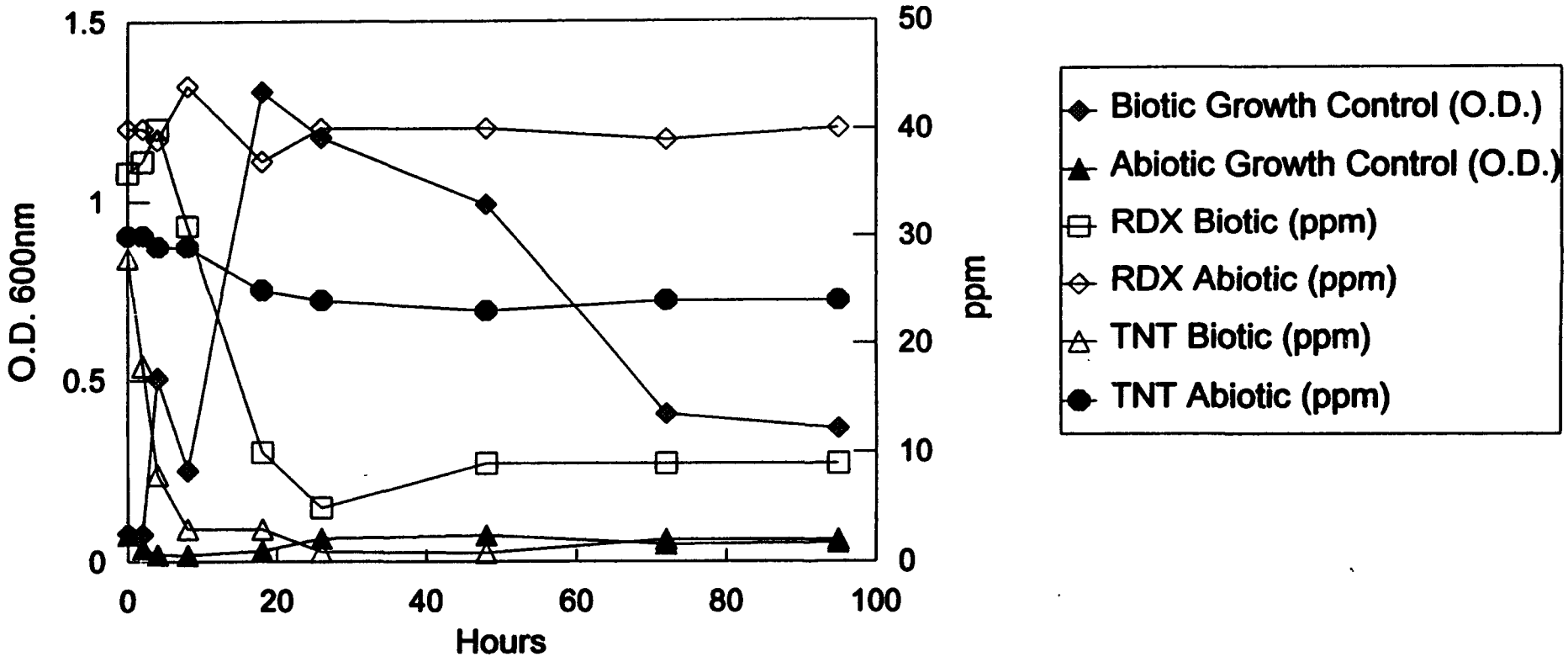

Figure 3. Growth curves of strain KMR-1 with conccomitant biotransformation of the explosives in brain-heart infusion medium reduced with cysteine. E_h = -210 mv. Cysteine = 0.08% (wt/vol).

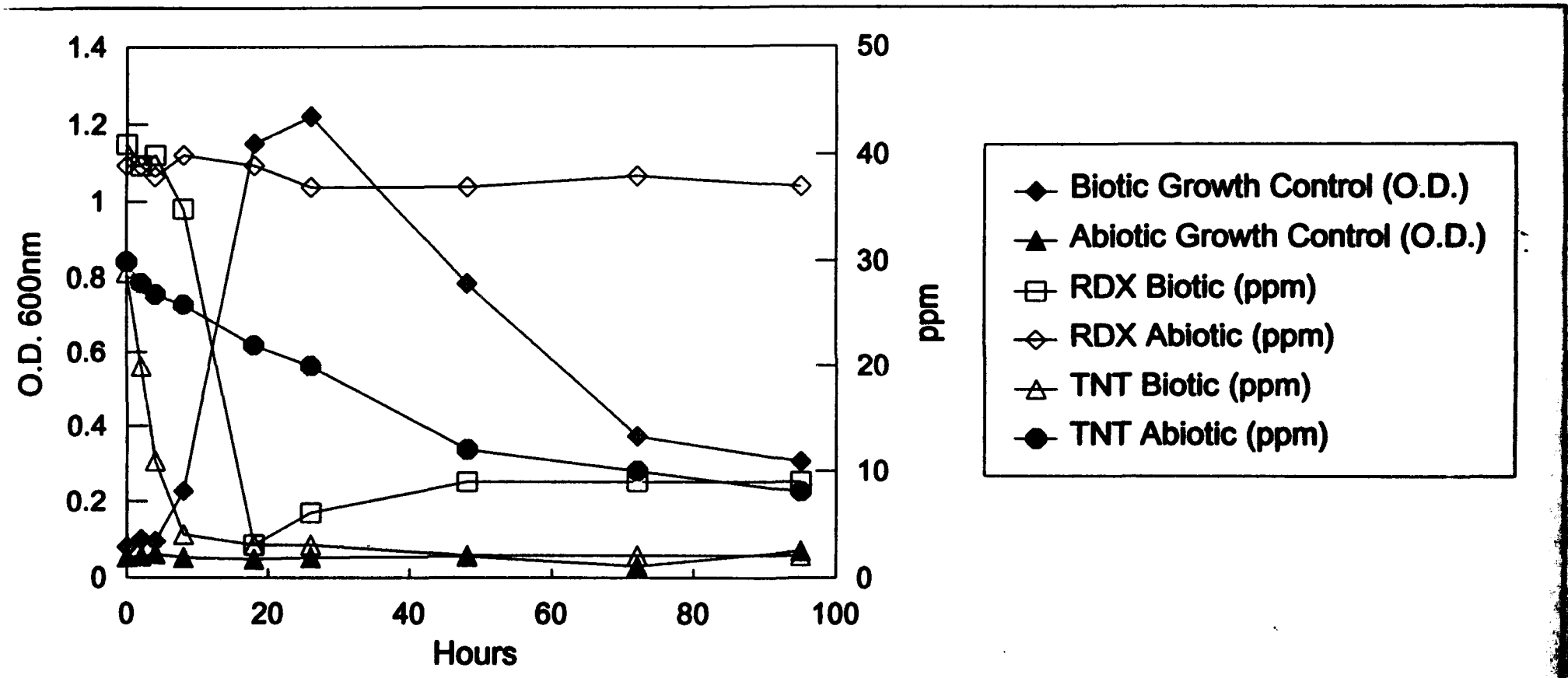

Figure 4. Growth curves of strain KMR-1 with concomitant biotransformation of the explosives in brain-heart infusion medium reduced with dithionite. E_h < -600 mv. Dithionite = 0.001% (wt/vol).

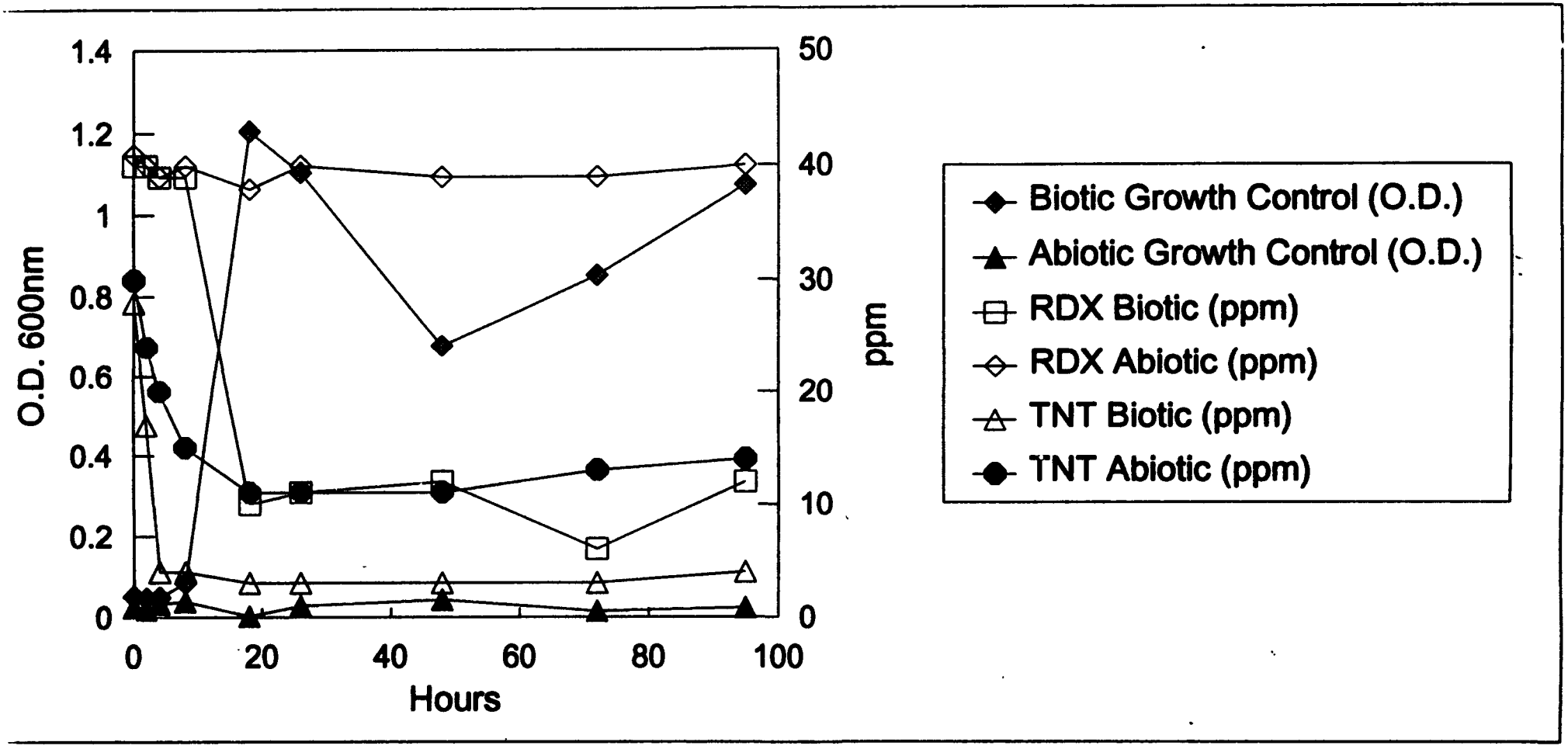

Figure 5. Growth curves of strain KMR-1 with concomitant biotransformation of the explosives in non-reduced brain-heart infusion medium. E_h was not determined.

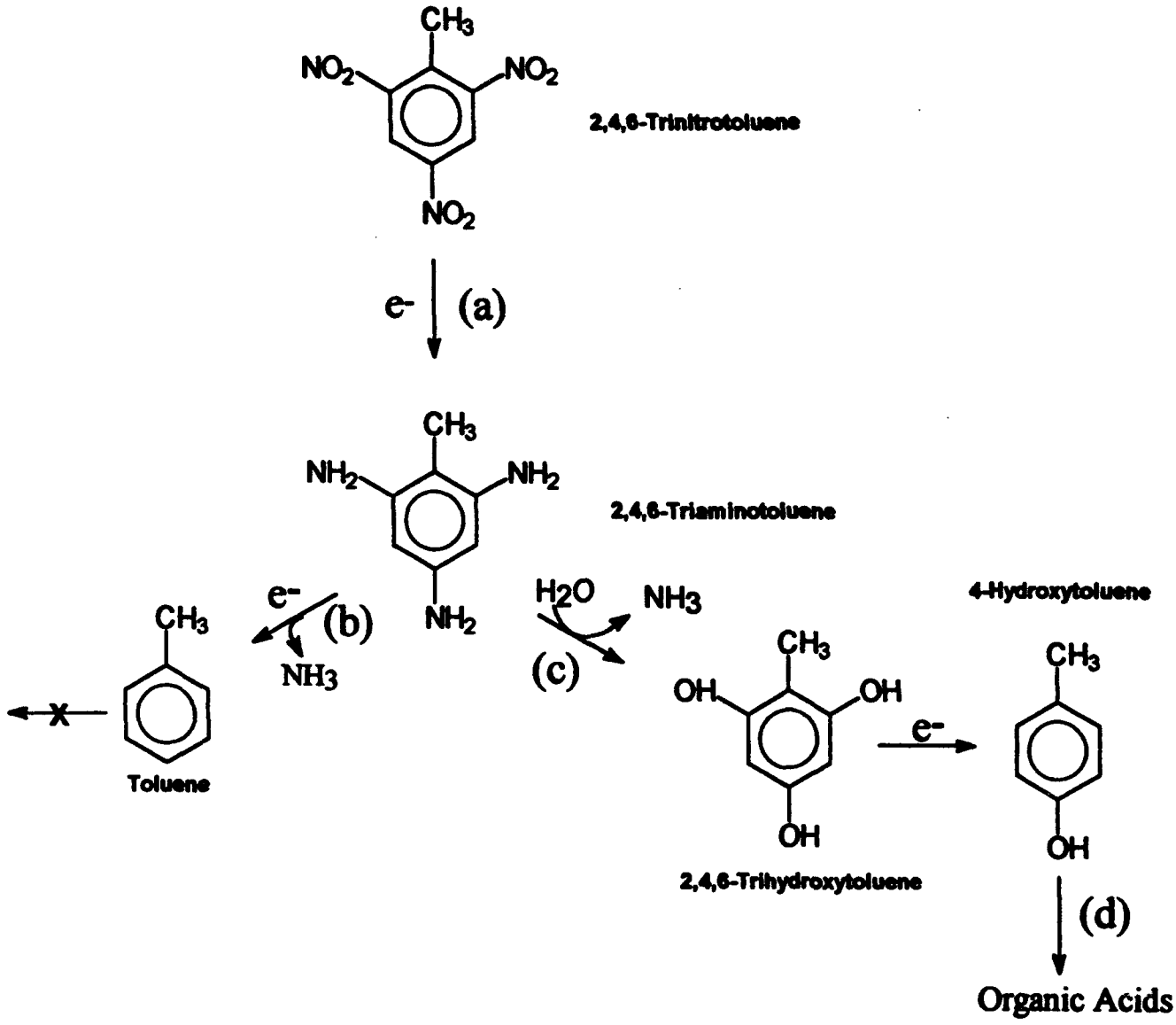

Figure 6. Degradation of 2,4,6-trinitrotoluene by strictly anaerobic bacteria: (a) Many steps, probably involving abiotic and biotic reactions; intermediates include nitroso- and hydroxylamine-derivatives; catalyzed by a variety of reductive enzymes (Preuss and Rieger, this volume); common in many anaerobes. (b) Observed in sulfate reducers. (c) Observed in *Clostridium* strains. (d) Probably employs traditional fermentative/reductive pathways.

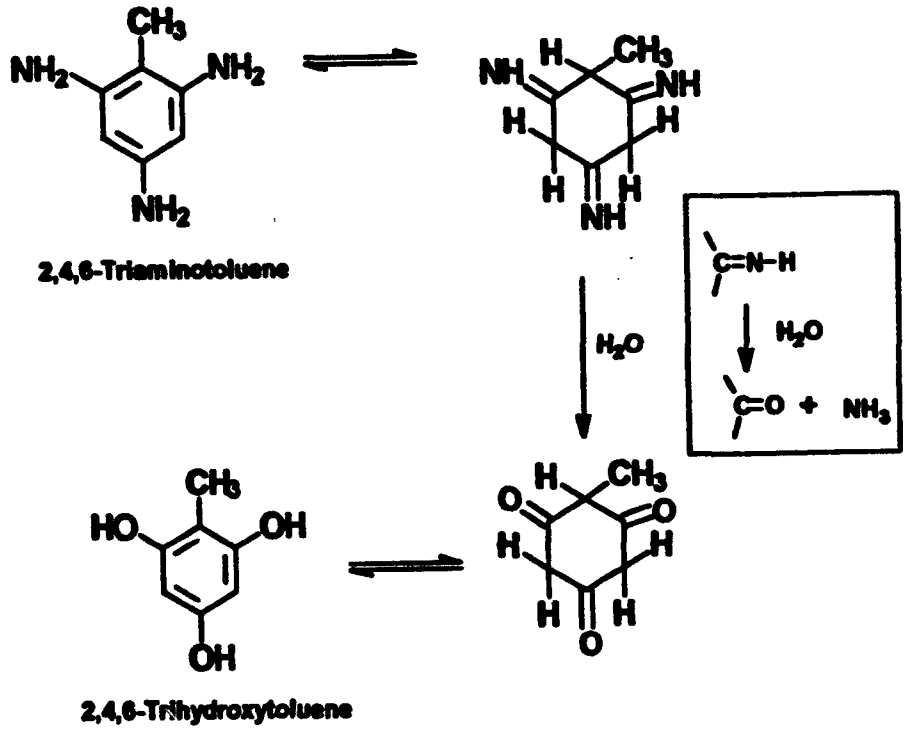

Figure 7. Proposed mechanism for non-enzymic conversion of 2,4,6-triaminotoluene to 2,4,6-trihydroxytoluene.